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Selective Detection of *Mycoplasma fermentans* by Polymerase Chain Reaction and by Using a Nucleotide Sequence within the Insertion Sequence-Like Element

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A new assay using the polymerase chain reaction to amplify a 206-nucleotide specific gene sequence within the insertion sequence-like element of *Mycoplasma fermentans* has been developed. The unique insertion sequence-like element exists in multiple copies in the *M. fermentans* genome. The assay selectively amplifies DNA from all strains of *M. fermentans* tested. In contrast, DNA from other species of human and nonhuman mycoplasmas, common tissue culture-contaminating mycoplasmas, and bacteria, as well as human, monkey, and mouse tissues do not produce the amplified DNA products specific for *M. fermentans*.

Mycoplasmas of the species *Mycoplasma fermentans* have recently been recognized as possible infectious pathogens in humans (4, 7, 9). Many patients with AIDS suffer a systemic infection caused by this microbe (5, 7). The role of this newly recognized mycoplasmal infection in the disease of AIDS, however, is still not understood. The microbe found in the immunocompromised patients may simply represent another opportunistic infection, play a cofactor role of promoting disease progression in AIDS (8), or actually be producing major pathogenesis in the chronic debilitating AIDS disease (1, 5).

In order to further investigate the mycoplasma's pathogenic role in human disease and to develop a productive culture system for isolating this apparently fastidious microorganism from clinical specimens, we needed a highly sensitive and specific technique for detection. We had previously developed a polymerase chain reaction (PCR) assay based on the specific nucleotide sequence found at one terminus of the cloned DNA psb-2.2 from the incognitus strain of *M. fermentans* (3, 7). Unfortunately, the reaction failed to detect some strains and clinical isolates of *M. fermentans*. The targeted sequence is apparently not present in all of the strains.

We have focused on applying newly available molecular genetic information to develop another highly sensitive and specific assay which may allow us to detect all strains of *M. fermentans*. In this study, we describe a new PCR assay based on the nucleotide sequence within the insertion sequence (IS)-like genetic element (3). This 1.4-kb unique element exists in multiple copies in the DNA of *M. fermentans* but not in other species of mycoplasmas. The new PCR assay using nucleotide sequences within the IS-like element was found to be highly specific and extremely sensitive.

Mycoplasma pneumoniae (ATCC 15531), *M. fermentans* (ATCC 19989), *Mycoplasma hominis* (ATCC 15488), *Mycoplasma orale* (ATCC 23714), *Mycoplasma genitalium* (ATCC 33530), *Mycoplasma arginini* (ATCC 23838), *Mycoplasma salivarium* (ATCC 23064), *Acholeplasma laidlawii* (ATCC 23206), *Ureaplasma urealyticum* (ATCC 27618), and *Mycoplasma hyorhinis* (ATCC 17981) were obtained from the American Type Culture Collection, Rockville, Md. *M. fermentans* (MT-2, K-7, and PG18 strains), *Mycoplasma iowae*, *Mycoplasma buccale*, *Mycoplasma pirum*, *Mycoplasma alvi*, *Mycoplasma sualvi*, *Mycoplasma moatsii*, *Mycoplasma arthritidis*, *Mycoplasma capricolum*, and *Mycoplasma pulmonis* were all kindly provided by J. G. Tully, National Institute of Allergy and Infectious Diseases. Two additional clinical isolates of *M. salivarium* and one clinical isolate of *M. orale* had been previously isolated in our laboratory from oral and pharyngeal swabs, respectively. Nine additional clinical isolates (three recently isolated in our laboratory from patients with AIDS and six isolated from non-AIDS patients with acute respiratory disease by R. Dular of Ottawa, Public Health Laboratory, Ottawa, Ontario, Canada) of *M. fermentans* will be reported in detail elsewhere. All the mycoplasmas were grown at 37°C in SP-4 medium. *Clostridium perfringens*, *Streptococcus pneumoniae*, and *Escherichia coli* were kindly provided by Ted Hadfield, Chief, Division of Microbiology, Armed Forces Institute of Pathology. The mycoplasmal DNAs were purified as previously described (6), and the concentrations of DNA stock solutions were determined by the spectrophotometric method (12). To obtain the desired concentration of DNA for the PCR assay, the DNA was serially diluted 10-fold in a solution containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.01% gelatin.

The amplification of selective DNA sequences was performed with thermostable recombinant Ampli-*Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) (11) in the automated Perkin-Elmer Cetus DNA thermal cycler. Each 10-μl DNA sample to be amplified was adjusted to a total volume of 100 μl with PCR buffer containing (final concentrations) 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 0.001% gelatin, 0.5 μM (each) primer RW004 and RW005, 200 μM (each) deoxynucleoside triphosphate, and 2.5 U of *Taq* DNA polymerase. The samples were then overlaid with 50-μl portions of mineral oil. Samples were denatured at 94°C for 2 min and 30 s and then amplified for 45 cycles. Each cycle consisted of denaturation at 94°C for

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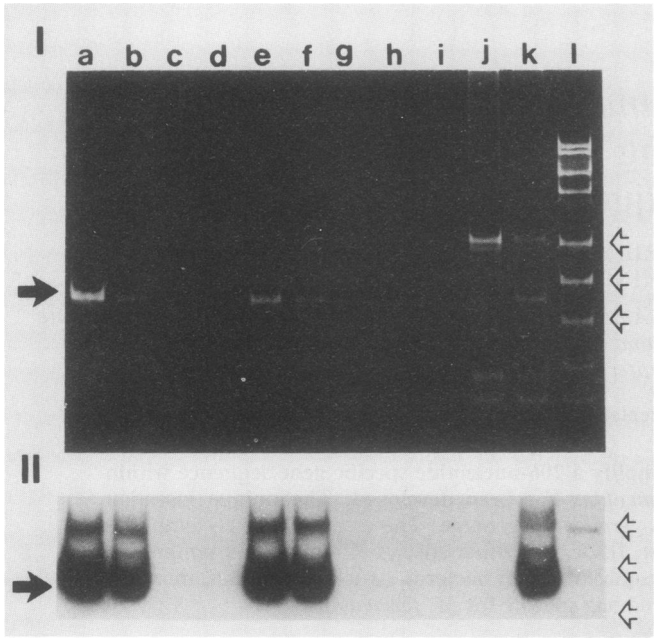
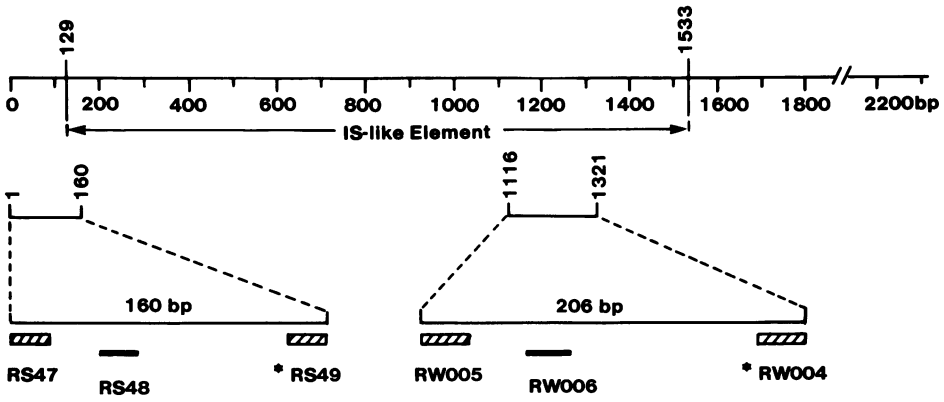


FIG. 1. Analysis of the PCR products from different strains of *M. fermentans* using primer pair RS47 and RS49. After amplification, a 20-μl aliquot from each sample is separated on a 6% polyacrylamide gel. The DNA is visualized by staining with ethidium bromide (I) and subsequently electroblotted to a nylon membrane and probed with ^{32}P-labeled RS48 (II). Lanes: a, c, e, and f, 10 fg of DNA from strain incognitus, PG18, K7, and MT-2, respectively; d, g, and h, 10 pg of DNA from PG18 and two of the three independent clinical isolates of *M. fermentans*, respectively; b, 1 fg of incognitus strain DNA; i, control for DNA dilution buffer; j, 1 μg of human placenta DNA; k, 10 fg of incognitus DNA plus 1 μg of placental DNA during DNA amplification; and l, *Msp*I-digested pUC18 DNA as the size markers. The size of the diagnostic DNA band in PCR products using this primer pair is 160 bp (solid arrow on the left). The open arrows to the right indicate the positions of relevant size markers of 242, 190, and 147 bp, respectively.

35 s, annealing of primers at 55°C for 45 s, and extension at 72°C for 50 s. The annealing time was increased by 1 s per cycle during amplification. After the final cycle, the annealing time was increased to 5 min, extension for 5 min was conducted.

Aliquots (20 μl) from each amplified sample were removed and analyzed on a 6% polyacrylamide gel in 1× Tris-borate-EDTA buffer (7). The gels were stained with ethidium bromide, and the DNA was visualized by UV fluorescence. For Southern hybridization analysis (13), the fractionated DNA was electroblotted onto a Zeta-Probe membrane (Bio-Rad Laboratories, Richmond, Calif.) at 60 V for 1 h in 0.5× Tris-acetate-EDTA buffer (7) with an electroblotting apparatus (Hoefer, San Francisco, Calif.), denatured, fixed in 400 mM NaOH–2 mM EDTA for 12 min at room temperature, and air dried for 10 min. The Zeta-Probe membrane was rinsed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) in 100 mM Tris-HCl (pH 7.5). Prehybridization was carried out in a solution containing 30% formamide, 4× SSC, 5× Denhardt's solution, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1% sodium dodecyl sulfate, and 350 μg of denatured salmon sperm DNA per ml at 42°C. Hybridization was done in the same mixture but containing the oligonucleotide probe RW006, which was 5′ end labeled with [γ-^{32}P]ATP, and was conducted overnight at 30°C (7). After hybridization, the membrane was washed at 37°C in 2× SSC–0.5% SDS three times (20 min each).

Primers (RS47 and RS49) of our previous PCR assay were chosen to detect *M. fermentans* by producing a specific amplified DNA fragment of 160 bp (7). Ten femtograms of DNA consistently yielded a positive 160-bp amplified band in DNA isolated from the incognitus strain of *M. fermentans*, from a strain (K7) previously isolated from the bone marrow of a patient with leukemia-lymphoma and from a strain (MT-2) isolated from a contaminated human lymphocyte culture (Fig. 1, lanes a, e, and f). In contrast, even a



Synthetic Primer and Probe		Sequence (5′-3′)
RW 004	Primer (24 nt)	GGA CTA TTG TCT AAA CAA TTT CCC
RW 005	Primer (24 nt)	GGT TAT TCG ATT TCT AAA TCG CCT
RW 006	Probe (24 nt)	GCT GTG GCC ATT CTC TTC TAC GTT

FIG. 2. Sequences of the synthetic oligonucleotide primer pair (RW004 and RW005) and the verification probe (RW006) used for specific DNA amplification of *M. fermentans* and their positions in the IS-like element. The relative positions of the primer pair RS47 and RS49 and probe RS48 used in our previous PCR assay are also indicated.

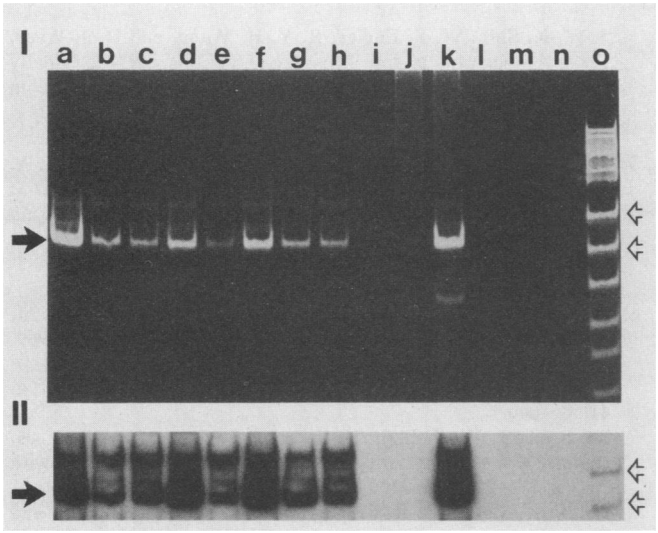


FIG. 3. Specific amplification of DNAs from various strains of *M. fermentans* using oligonucleotide primer pair RW004 and RW005. After gel electrophoresis, the DNA is visualized by ethidium bromide staining (I) and subsequently by Southern hybridization with ^{32}P-labeled RW006 probe and autoradiography (II). Lanes: a, 10 fg of incognitus DNA; b, 1 fg of incognitus DNA; c, 1 fg of PG18 DNA; d, 10 fg of PG18; e, f, g, and h, 1 fg of DNA from K-7, MT-2, and two independent clinical isolates, respectively; i, control for DNA dilution buffer; j, 1 μg of placental DNA; k, 1 fg of incognitus DNA plus 1 μg of placental DNA during amplification; l, m, and n, 1 ng of DNA from *M. pirum*, *M. pneumoniae*, and *M. hominis*, respectively; and o, *Msp*I-digested pUC18 DNA as the size markers. The size of the diagnostic DNA band is 206 bp (solid arrow on the left). The open arrows to the right indicate the positions of the two relevant size markers of 242 and 190 bp.

1,000-fold higher amount of DNA (10 pg) isolated from the prototype strain of *M. fermentans* (PG-18 and ATCC 19989) as well as DNA from two recent clinical isolates from human immunodeficiency virus-positive patients with AIDS (2) tested negative for the diagnostic DNA fragment (Fig. 1, lanes d, g, and h).

We recently identified the presence of multiple copies of an IS-like genetic element in *M. fermentans* (3). The actual copy number of the IS-like element found in the genomes of different strains or isolates of *M. fermentans* may vary and range from 5 to more than 10 copies (3). We have redesigned our PCR assay on the basis of specific nucleotide sequences which are located within the IS-like element (Fig. 2). A new set of primers (RW004 and RW005) was chosen to produce a specific amplified fragment of 206 bp in our new PCR assay. The nucleotide sequences of RW004 and RW005 are shown in Fig. 2.

Using the new set of primers, we could consistently detect 1 fg of DNA in all strains of *M. fermentans* tested in the assay (Fig. 3, lanes b, c, and e to h) including the prototype strain PG-18 and the new clinical isolates from patients with AIDS, whose DNA (up to 10 pg) tested negative in the PCR reaction with the old set of primers. Sensitivity of this newly developed PCR assay was further verified by successfully detecting 1 fg of the *M. fermentans* DNA in the presence of 1 μg of nonspecific human background DNA (Fig. 3, lane k). The presence of higher-molecular-weight bands in the positive reaction is most likely due to the presence of single-stranded DNA products or to a slight variation among the multiple copies of the IS-like element in the mycoplasmal genome. However, this only occurs in the DNA producing a positive reaction and does not affect the positivity of results. Specificity of the reaction has also been examined by attempting to amplify the DNAs isolated from other human and nonhuman mycoplasmas, common tissue culture-contaminating mycoplasmas, gram-positive and gram-negative bacteria, mouse, monkey, and human cell culture and tissue. PCR amplification of these DNAs did not produce the specific 206-bp DNA fragment and there was no hybridization with 5′-end-labeled oligonucleotide RW006 on subsequent Southern blot analysis (Table 1).

TABLE 1. Specificity of PCR for *M. fermentans* using unique sequences within the IS-like genetic element

Source	Concn of DNA tested	Positivity
Mycoplasmas		
M. fermentans		
ATCC 19989	1 fg	+
incognitus strain	1 fg	+
PG-18	1 fg	+
K-7	1 fg	+
MT-2	1 fg	+
and each of nine additional clinical isolates	1 fg	+
M. hominis (ATCC 15488)	1 ng	−
M. orale (ATCC 23714)	1 ng	−
and one additional clinical isolate	1 ng	−
M. salivarium (ATCC 23064)	1 ng	−
and each of two additional clinical isolates	1 ng	−
M. buccale	1 ng	−
M. pneumoniae (ATCC 15531)	1 ng	−
M. genitalium (ATCC 33530)	1 ng	−
M. arginini (ATCC 23838)	1 ng	−
M. pirum	1 ng	−
M. alvi	1 ng	−
M. moatsii	1 ng	−
M. sualvi	1 ng	−
M. iowae	1 ng	−
M. arthritidis	1 ng	−
M. capricolum	1 ng	−
M. pulmonis	1 ng	−
M. hyorhinis (ATCC 17981)	1 ng	−
Acholeplasma laidlawii (ATCC 23206)	1 ng	−
Ureaplasma urealyticum (ATCC 27618)	1 ng	−
Bacteria		
E. coli	1 μg	−
Streptococcus pneumoniae	1 μg	−
Clostridium perfringens	1 μg	−
Mouse		
NIH/3T3	1 μg	−
Spleen (BALB/c)	1 μg	−
Liver (BALB/c)	1 μg	−
Brain (BALB/c)	1 μg	−
Monkey		
Vero cells (ATCC CCL18)	1 μg	−
Spleen (green monkey)	1 μg	−
Liver (green monkey)	1 μg	−
Brain (green monkey)	1 μg	−
Human		
CCRF-CEM (ATCC CCL119)	1 μg	−
Each of 4 Placentas (normal delivery)	1 μg	−
Each of 50 PBMC[a] (normal donor)	1 μg	−

[a] PBMC, peripheral blood mononuclear cells.

The present study shows that we have developed a highly selective assay to detect *M. fermentans* by PCR with remarkable sensitivity. We have taken advantage of the presence of multiple copies of a unique IS-like genetic element in *M. fermentans* DNA. The nucleotide sequence of the targeted segment as well as the primers is located within the IS-like element. The assay detects all the different strains including the new clinical isolates of *M. fermentans* which our previous PCR assay failed to detect. Furthermore, the limitation of reaction sensitivity per assay for our current PCR assay is 0.1 to 1 fg of *M. fermentans* DNA within a background of 1 μg of human DNA instead of the 1 to 10 fg of microbe DNA in our previous PCR assay. Since the genome size of mycoplasmas is approximately 1,000 kb (10), 1 fg of DNA is essentially equivalent to the amount of DNA derived from a single organism. Thus, a molecular technique selectively detecting a single microorganism of *M. fermentans* in a reaction is readily available. We believe the information and the technique described here can be very useful and should significantly facilitate the studies of this AIDS-associated mycoplasma in the disease of AIDS.

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